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THE INFECTION PROCESS OF *Fusarium subglutinans* IN *Pinus merkusii* SEEDLINGS

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ABSTRACT

Pinus merkusii or tusam is an original Indonesian plant and it is naturally distributed in Aceh and North Sumatra. *Damping-off* disease is the main problem in its nurseries. *Fusarium subglutinans* is one of the leading causes of *damping-off* disease. The knowledge of fungal infections process of tusam seedlings is essential to control *damping-off* disease effectively.The aim of this research is to understand (1) infection process of *F. subglutinans* in tusam seedlings and the defence response of seedlings against the infection of *F. subglutinans*. The methods used in this research were (1) identification of fungal pathogens that causing the disease, (2) pathogenicity test of *F. subglutinans*, (3) detection the accumulation of lignin, accumulation of callose and hypersensitive reactions by staining of seedling tissue using phloroglucinol, aniline blue and lactophenol trypan blue.The results of this study revealed that spores germination occurred in two days after inoculation. Direct penetration through cell wall and stomata was observed on the third day after inoculation. There was hypersensitive reaction in stomata. Accumulation of callose and lignin appeared on the third day after inoculation. However, defence response of seedlings was not effective, as *F. subglutinans* is a necrotroph fungus.

Keywords: damping-off, *Fusarium subglutinans, Pinus merkusii,* tusam

INTRODUCTION

The increasing demand for timber and the reduced natural forests area in Indonesia is the reason for the government to develop industrial plantation forest (HTI) as an alternative to meet the needs of the timber industry. *Pinus merkusii* here is referred to as tusam, has high economic value due to the variety of its utilization such as for lightweight construction, furniture, pulp, matches and chopsticks. In addition, it produces turpentine. A single tree can produce 20-40 kg pure resin and 7-14 kg turpentine anually (Hidayat and Hansen, 2001).

Quality of seedlings in the nursery is a fundamental to the success of planting tusam. One of the current problems in tusam nursery is the damage due to *damping-off* disease. *Fusarium* spp. is the most common pathogen giving rise to *damping-off* in the nursery of tusam (Widyastuti, 1996). One of them is *F. subglutinans* which is facultative parasite, it lives in the soil and other organic materials as a saprophyte and switches into parasite when environmental condition is favourable. Control of diseases caused by *Fusarium* sp. is quite difficult as the pathogens are soil inhabitant which is able to survive in the soil for a long period of time (Susanti *et al*., 2009).

The infection process of *F. subglutinans* needs to be investigated to understand the defence response of tusam seedlings. Both are essential to achieve effective control of *damping-off* disease in tusam nursery.

MATERIALS AND METHODS

The seedling of tusam (*P. merkusii*) and pathogenic fungi *F. subglutinans* isolated from *P. merkusii* showed symptom of *damping-off* used in this study. The research procedure is described in Figure 1.

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Identification of Fungal Pathogen

Identification of fungal pathogen was conducted by observing the symptoms and signs of infection appearing in host plant. Pathogenic fungi attacking tusam seedling was isolated according to a procedure developed by Salerno and Lory (2007). Stem tissue of infected seedlings showed fungal mycelium of 0.5-1 cm cut with a scalpel and sterilized with soaked into 2.5% klorox (sodium hipochlorite) for 20 seconds and into 70% ethanol for 20 seconds. Materials were rinsed with sterile water and dried with sterile filter paper, before laid on Potato Dextrose Agar (PDA) medium.

The character of fungal pathogen was observed daily along with the diagonal growth of isolates on Petri plate (diameter 9 cm) until the medium grew. Hyphae character and spores were also observed.

In Planta Pathogenicity Trial

To investigate the pathogenicity of *F. subglutinans* to tusam seedling, the healthy seedling was inoculated with *F. subglutinans*. Seedling materials were collected daily, starting on the second day to the sixth day or when all the seedlings collapsed.

Seedling materials were immersed in 96% ethanol for 24 hours and boiled for five minutes, then were kept at room temperature for 24 hours. Materials were then soaked into 20 ml of 2.5 g/ml chloral hydrate solution (Merck). Chloral hydrate solution was served to accelerate transparency of tissue to facilitate the observation. Microscopic observations were performed using Olympus microscope BX 51 series, Olympus DP 70 camera and DP controller software.

Trial of Tusam Seedling Defense Response

To understand the defence response of tusam seedling, another trial with similar initial treatment as previous trial was conducted. In this trial defence response of tusam seedling was studied by conducting microcopic observation of tusam seedling tissue with staining technique prior to the observation.

Three different microbiological staining colors were applied in this trial, i.e. (1) lactophenol trypan-blue to observe the structure of fungal pathogen within host plant tissue and to detect hypersensitivity reaction of plant cells, (2) aniline blue to detect the accumulation of kalose, (3) phloroglucinol to detect the accumulation of lignin.

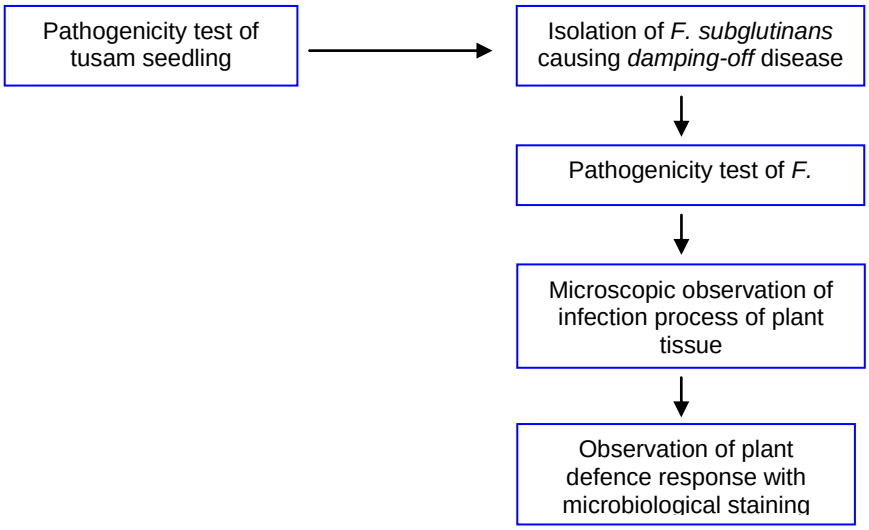

Figure 1. Research Procedure

RESULTS AND DISCUSSION

Identification of Fungal Pathogens

In the macroscopic observation, the affected seedling showed sign and symptom of *damping-off* (Figure 2). The figure shows the tissue of tusam seedling was covered with white mycelium which proved the presence of fungal infection. The suspected fungus causing *damping-off* was isolated (Figure 3). The isolate of *F. subglutinans* was found to overgrow on PDA medium plate on day twelve. The color of colony of *F. subglutinans* was white at the beginning then it turned into purple when it grew older (Leslie and Summerell, 2006; Viljoen *et al*., 1997). According to the macroscopic and microscopic identification, the fungal pathogen causing *damping-off* on tusam seedling was confirmed as *F. subglutinans*.

Figure 2. Tusam sedlings in the macroscopic observation **(a)** Tusam seedling in 6 days after inoculation of *Fusarium subglutinans,* showing *damping-off* symptoms with fungal pathogen mycelium infecting the seedling **(b)** Six-day-old healthy seedling of tusam.

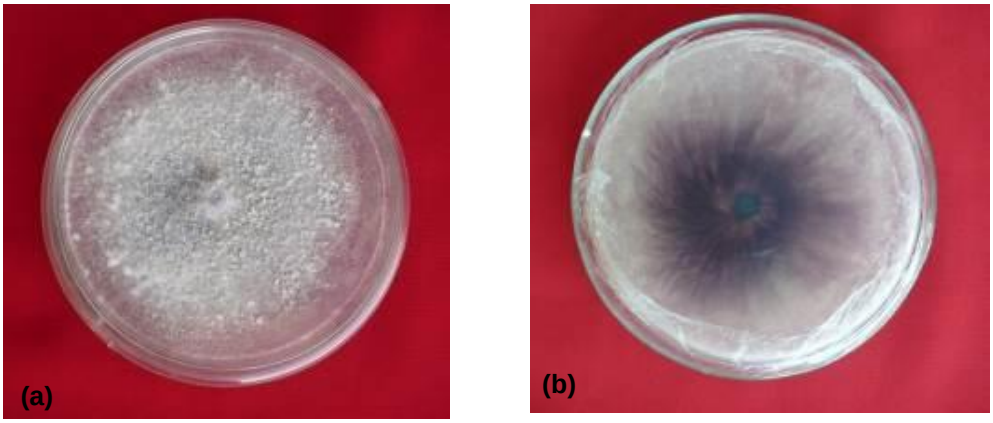

Figure 3. Six-day-old pure isolate of *Fusarium subglutinans* grown in PDA medium **(a)** Upright view of Petri plate **(b)** Purple mycelium, observed from the bottom of Petri plate

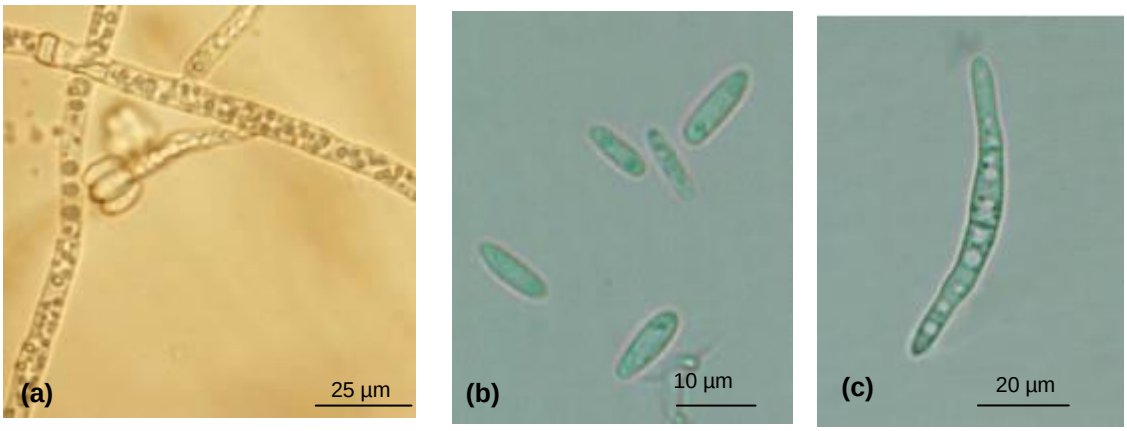

Figure 4. Conidia of *Fusarium subglutinans*; **(a)** in situ microconidia (arrow); **(b)** microconidia; **(c)** macroconidia.

The presence of special characters i.e. in situ microconidium and clamidospora, as well as the rarity of macroconidia confirmed that this pathogen was *F. subglutinans* (Leslie and Summerell, 2006). The character of in situ microconidia of *F. subglutinans* is shown in Figure 4a while Figure 4b and 4c showed macroconidia and 2-3 septate microconidia. Microconidia were present abundantly with an average size of 5-12 x 2.2 to 3.5 µm. They are present in a very small amount with an average size of 25-45 x 3 to 4.5 µm. In situ microconidia are oval-shaped with diameter of 5-15 µm.

Pathogenicity test of *Fusarium subglutinans*

Pathogenicity test was conducted to confirm that the fungal isolated from *damping-off* seedling was the same as fungal pathogen causing disease in tusam seedling. The result of the test is presented in Figure 5.

The pure cultures of pathogenic fungi inoculated on two-day-old healthy seedlings, causing the seedling to be damaged signed by similar symptoms to the previous *damping-off* seedling. Fungal pathogen was isolated from *damping-off* seedling and laid on PDA media. The fungi reisolated from infected seedling showed similar characters of conidia and mycelium to previous isolated fungal pathogen (Figure 8c and 8g). The symptoms presented on healthy tusam seedlings inoculated with pathogenic fungi showed that the fungi infecting the seedling were pathogen and caused *damping-off* of tusam seedling.

Pathogens *F. subglutinans* caused the seedling to die in seven days after inoculation. This is presumably because *F. subglutinans* produced secondary metabolites which are pathogenic and accelerated cell the death by causing root to rot in the seedlings and plants (Carlile *et al*., 2001).

Pathogenicity test of *Fusarium subglutinans* in tusam seedling

Fusarium subglutinans is a necrotrophic fungi (Viljoen *et al*., 1997) mostly causing rot disease in host plants (Okubara and Paulitz, 2005). This type of fungi infects host plant by directly penetrating into the plant tissues, killing the cells and using the nutrients present in the host plant for growth (Carlile *et al*., 2001). On the contrary, the biotrof parasites suck nutrients of living cells of host plant using their houstorium. Host plants infected with biotrof fungi usually survive for a longer time compared to plants with nekrotrof fungi (Semangun, 2001).

The observation of infection process was conducted to study the mechanism of infection process of *F. subglutinans* inoculated on tusam seedlings, particularly in the seedling stem. The process of infection was observed in inoculated *F. subglutinans* on tusam seedlings. The macroscopic and microscopic observation of *F. subglutinans* infection process on tusam seedlings and as well as observation on a

healthy tusam seedling as controls are presented in Figure 6. Process of *F. subglutinans* infection on tusam seedling stem began on the second day after inoculation when the spores of *F. subglutinans* started to stick on the seedling stem surface. These circumstances allow fungal hyphae to quickly germinate and penetrate the stem tissue.

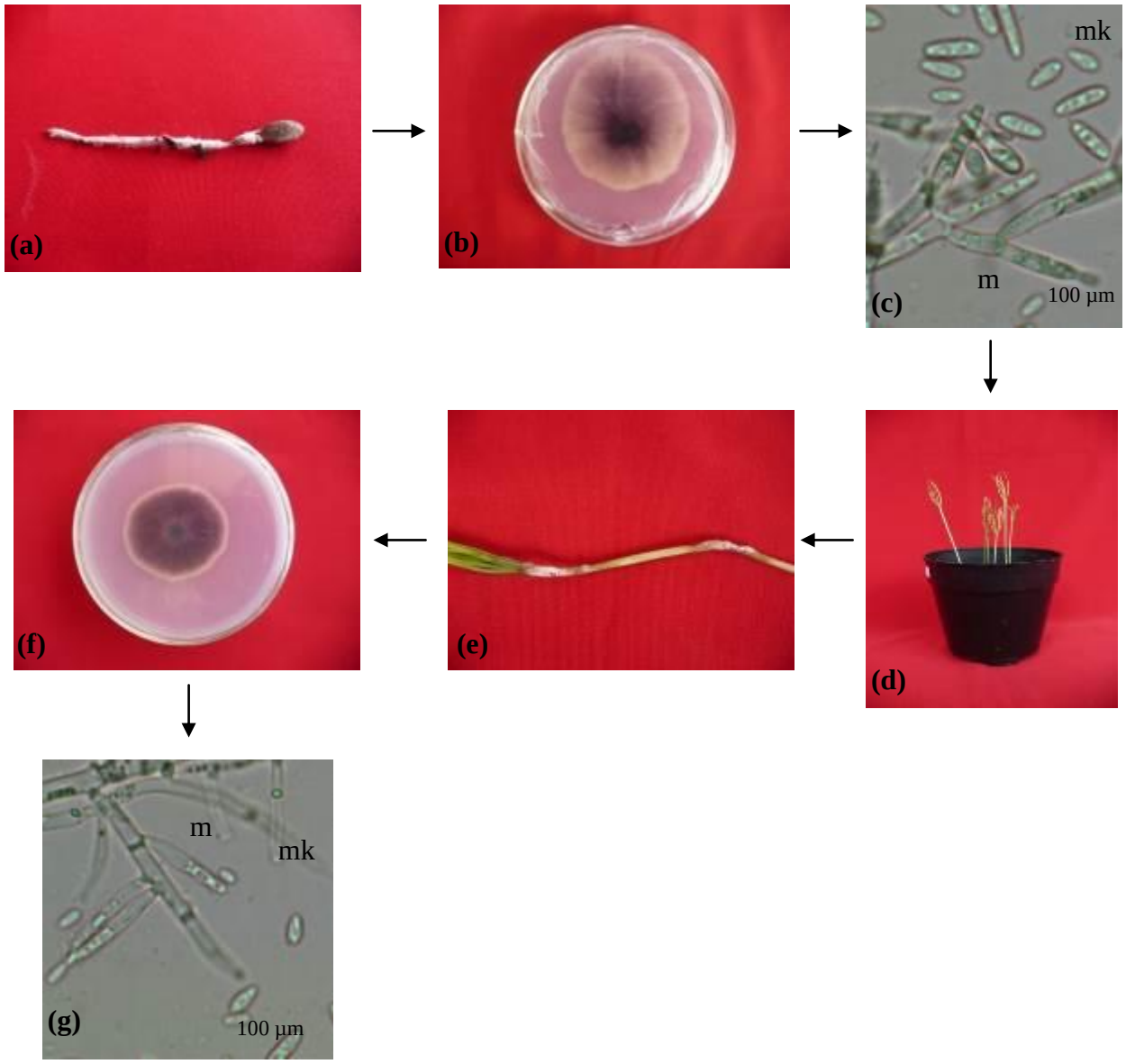

Figure 5. Pathogenicity trial of *Fusarium subglutinans*; (a) *damping- off* seedling of tusam (*Pinus merkusii*); (b) five-day-old fungal pathogen isolate; (c) conidia and mycelium of fungal pathogen isolated from *damping-off* seedling; (d) fungal pathogen isolated from *damping-off* seedling inoculated on the healthy tusam seedling; (e) inoculated tusam seedling showing *damping-off* symptom in 5 days after inoculation; f) re-isolated fungal pathogen; (g) conidia and mycelium of re-isolated fungal pathogen; (mk) micro conidium and (m) mycelium

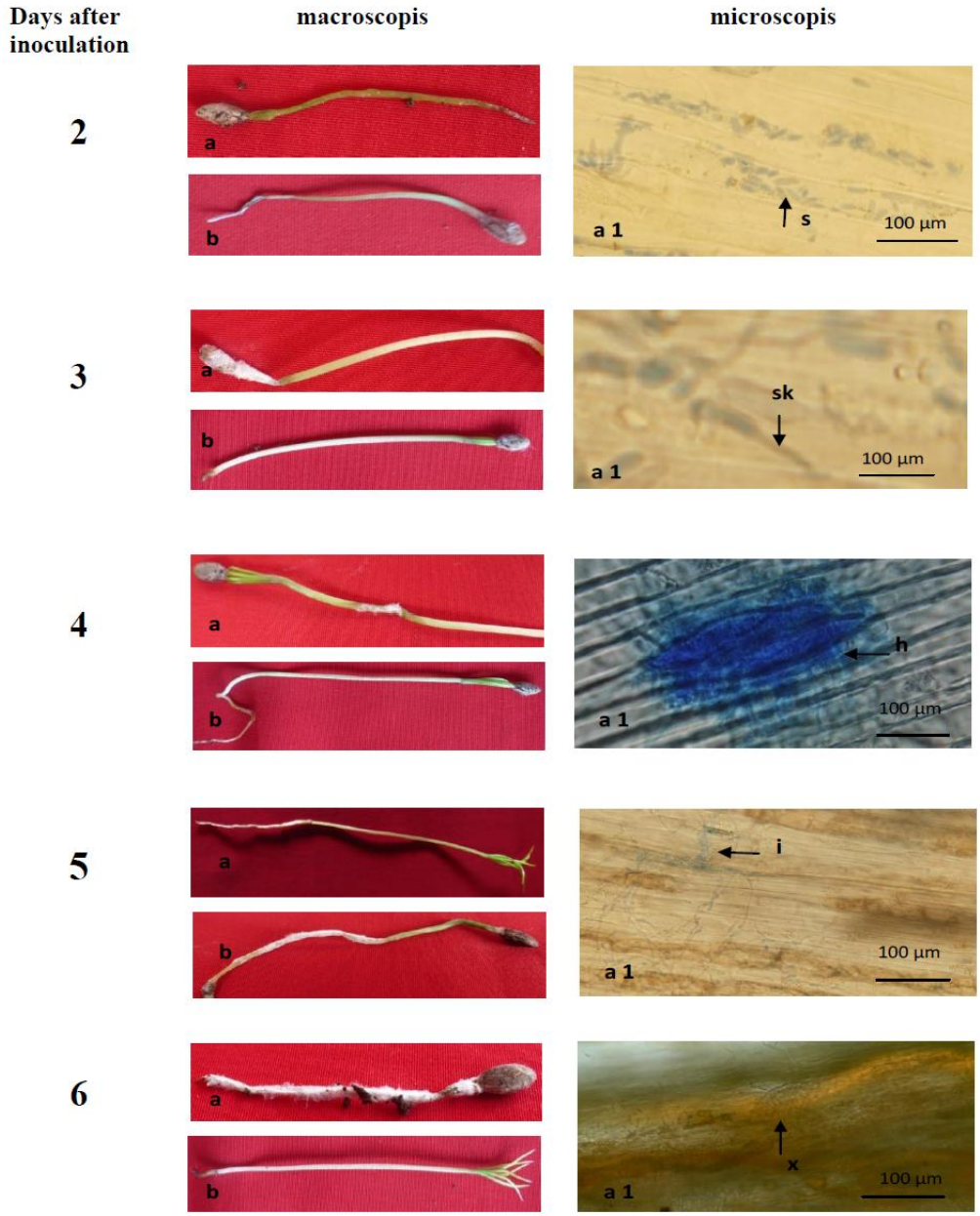

Figure 6. Pathogenicity of *Fusarium subglutinans* in tusam (*Pinus merkusii*) seedling; (a) infected tusam seedling; (a1) longitudinal crosssection of infected tusam seedling; (s) *F. subglutinans* spores; (sk) the germinated spore *F. subglutinans*; (h) hyphae entering stomata; (i) hyphae growing inside the extracelluler space in the vascular tissue (x); (b) healthy tusam seedling (control).

In day tree after pathogen inoculation, hyphae started to germinate on the surface of tusam seedling stem. In this phase hyphae of *F. subglutinans* were ready to penetrate into the host plant tissue for subsequent infection process. On day four after inoculation, the hyphae of *F. subglutinans* penetrated into plant tissue through stomata. The result of this study is corresponding to a similar study by Widyastuti *et al*. (2013) that revelead *F.subglutinans* also penetrated through stomata in the seedlings of *Acacia mangium*.

The comparison of stomata of healthy tusam seedlings with stomata of infected seedlings is presented in Figure 7. Stomata represent natural pores of plant used to exchange the gas when transpiration takes place in plants. Plants open and close the stomata naturally and in the opening of stomata, pathogen *F. subglutinans* enters seedling tissue. Stomata are commonly found on the leaves and stems of seedlings or young plants. Infected tusam seedlings (Figure 7b), showed accumulation of dark blue lactophenol trypan-blue. The accumulation of dark blue color indicates that the hyphae of *F. subglutinans* had entered tusam seedling tissue. Blue accumulation was present as the pathogen was capable of absorbing lactophenol trypan-blue.

In addition to penetration through the stomata, pathogen can penetrate directly into the tissue and also penetrate the protective layer, as shown in Figure 8a. The figure shows the comparison of hyphae of *F. subglutinans* entering through the stomata of the infected pine seedlings. According to Figure 8b, it was assumed that seedling tissue made effort to respond to the infection by hypersensitivity reactions to prevent the pathogen to grow inside the seedling tissue. It was presumably not effective, as pathogen had grown rapidly inside the tissue.

Pathogens were also capable of penetrating directly through the existing wound on the tissue surface caused by physical injury. Pathogens entering the tissue through wound due to injury were not found in this study, as the tested materials were planted in the pot covered with plastic warp. This treatment led to a very small possibility of physical injury to the seedling due to the lack of contact with wind, water, insects and human activity.

In five days after inoculation, the macroscopic observation to the seedlings revealed that white mycelium covered most of the seedling stems, and they started to collapse. The microscopic observation revealed that pathogen appears to infect the vascular tissue in the stem and presumably supressed almost all the biochemical activities occurring in the cells. It was proven by the presence of significant amount of hyphae and spores, both microconidia and macroconidia inside the seedling tissue. Under normal conditions as shown in control seedling, the first leaves of seedlings would emerge on day four or five (Figure 5).

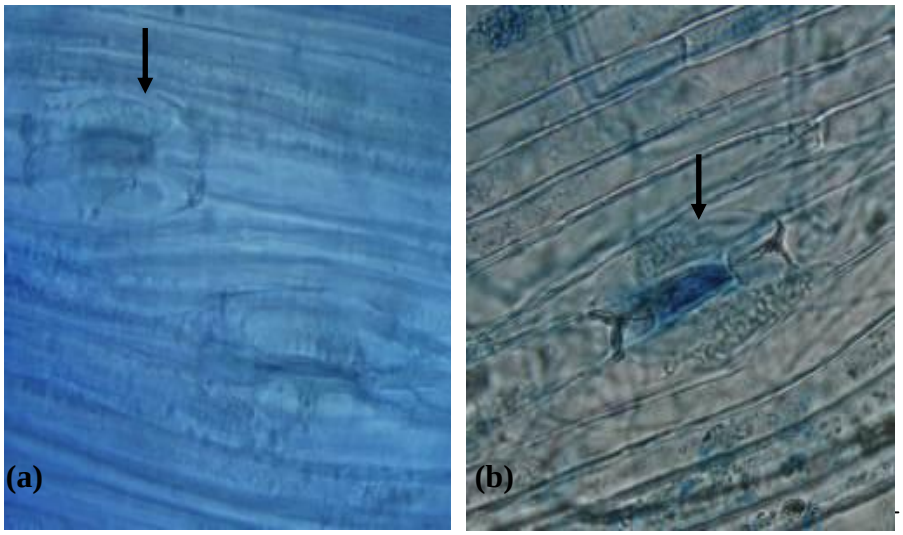

Figure 7**.** Stomata of tusam (*Pinus merkusii*) seedling stem **(a)** stomata of healthy seedling (arrow) **(b)** stomata of *Fusarium subglutinans* infected seedling, marked by dark blue color (arrow)

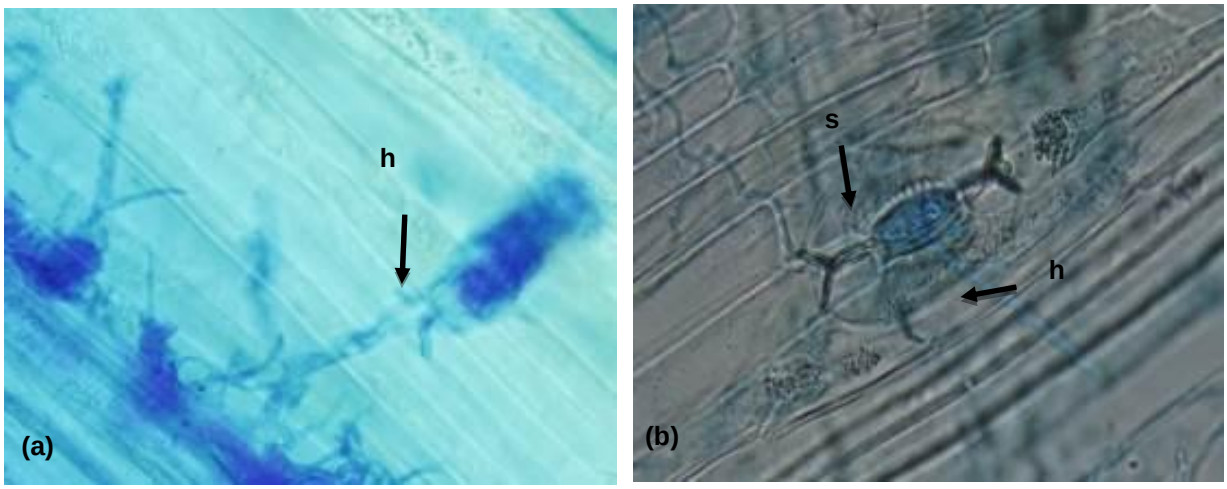

Figure 8. Penetration of *Fusarium subglutinans* into tusam (*Pinus merkusii*) seedling (a) Direct penetration of hyphae of *F. subglutinans* into tissue of tusam seedling; (b) Penetration of *F. subglutinans* hyphae into tusam seedling tissue; (h) hyphae of *F. subglutinans*; (s) infected stomata

The observation on seedling was terminated on day 6 after inoculation when the most of the seedlings collapsed and died. Visually, most of their stems were covered with fungal mycelium. The seedlings become very brittle due to decaying. The microscopic observation revealed that the entire tissue seedlings were filled with hyphae and the color of cross-section of tissue turned into dark brown as a sign of cell death (Figure 5).

Under microscopic observation, the infection process of *F. subglutinans* on tusam seedling did not occur simultaneously. Generally, on the fifth day, the hyphae of *F. subglutinans* filled the seedling tissue, but the germinating spores or spores in the penetration phase through open stomata were also found. It presumably occurred due to the varying development of spores and hyphae. The condition would rapidly evolved if environmental conditions were suitable.

Defence Response of Tusam Seedling

Defence response of tusam seedling against fungal *F. subglutinans* attack was conducted by observation on infected seedling tissue. Staining was carried out prior to the observation to facilitate the ease of observation and evaluation of the resistance response. The dye used in this study was phloroglucinol and aniline blue.

Microscopic observation on phloroglucinol stained tissue was conducted to detect lignin accumulation in seedling tissues. Lignin has been widely studied as it plays an important role in host plant resistance against pathogenic attack (Bhuiyan *et al*., 2009). Lignification is an important mechanism in the defence response i.e. accumulating lignin in the cell wall around the injured area of pathogen infection. Lignification in the cell wall will inhibit the development of pathogens by several mechanisms: (1) increasing the mechanical resistance of the host plant cell wall, (2) reducing the susceptibility of cell wall to extracellular enzymes, (3) limiting the diffusion of pathogenic compounds produced by fungal pathogens, (4) inhibiting the growth of pathogens by the presence of lignin precursors in the form of phenolic compounds which are toxic to pathogens (Kuc, 1983).

The microscopic observation of seedling tissue stained with phloroglucinol revealed a very thin lignin accumulation on the tissue especially around the site of infection (Figure 9). The presence of lignin accumulation was detected by orange color line and deposit. The lignification was not visible on day two after inoculation since the spores of *F. subglutinans* were at phase of

attach to the surface of the stem. Lignification was visible on the third day after inoculation when the hyphae of pathogen penetrated the tissue through stomata and the protective layer. Accumulation of lignin became increasingly apparent on the fourth and fifth day after pathogen inoculation. On the sixth day after inoculation, the plant tissue was damaged, the stem turned into brown and rotted. In this condition the production of lignin was decreased and the accumulation of lignin was no longer visible.

The thin deposition of lignin in tusam seedling infected by *F. subglutinans* was presumably because the seedlings were too young when attacked by pathogens. The process of lignin formation is closely related to the peroxidase activity (Hasegawa *et al*., 2005). Peroxidase is one of the enzyme associated with the biosynthesis of lignin from its monomer. High peroxidase activity triggers the formation of lignin. It is directly proportional to the hardening of stem. The older the plant, the harder stem and the more lignin will be available. This condition led to the increasing of defense response of plant against pathogen attack.

Staining with aniline blue is done to detect the presence of callose accumulation on seedling tissue. Callose accumulation was polymeric β-1,3-glucan, a component in a very small amount in healthy plant tissue depositing around the site of infection (Vance *et al*., 1980). The presence of callose accumulation is a part of the induced resistance in injured or infected plant. Generally callose deposition occurs when plant interacts with pathogens, in both susceptible and resistant plants. Callose accumulation contains compounds such as phenolic capable of blocking the attack by pathogen (Valluri and Soltes, 1990).

The observations using fluorescence microscope revealed that callose started to deposite on the third day after inoculation (Figure 10). The figure shows thin callose deposition surrounding penetrated site in the seedling tissue, while the observation using a standard microscope was only able to visualise the hyphae of *F. subglutinans* inside the tusam seedling tissue. Callose accumulation was not visible on the second day after inoculation, as the tissue was too young. In the non-infected seedling the defence response was not present. Six day after inoculation, the tissue was dead causing calose accumulation to disappear in the seedling.

A complete illustration of *F. subglutinans* infection process is visualized in Figure 11. Infection process begins when the *F. subglutinans* spores were attached to the surface of tusam seedlings. Once the spores were attached to the tissue, they would germinate and penetrate into the plant tissue in two different ways, i.e. direct penetration and stomatal penetration. The penetration of pathogens into the plant tissue triggered defense response of tusam seedlings. Defense response involved (1) hypersensitivity reaction characterized by a dark blue color in the microscopic observation with lactophenol trypan blue stain, (2) accumulation of lignin, which is characterized by the presence of orange color on microscopic observation of the tissue stained with phloroglucinol, (3) accumulation of callose characterized by the presence of blue color deposite on fluorescent microscopic observation of the tissue stained with aniline blue. When the plant immune system was incapable of inhibiting the growth of pathogens, the pathogens would continue to expand inside the tissues and cause the tissue to die. In this study, defense response of tusam seedlings failed to inhibit infection of *F. subglutinans*, resulting the death of the seedling on the sixth day after inoculation.

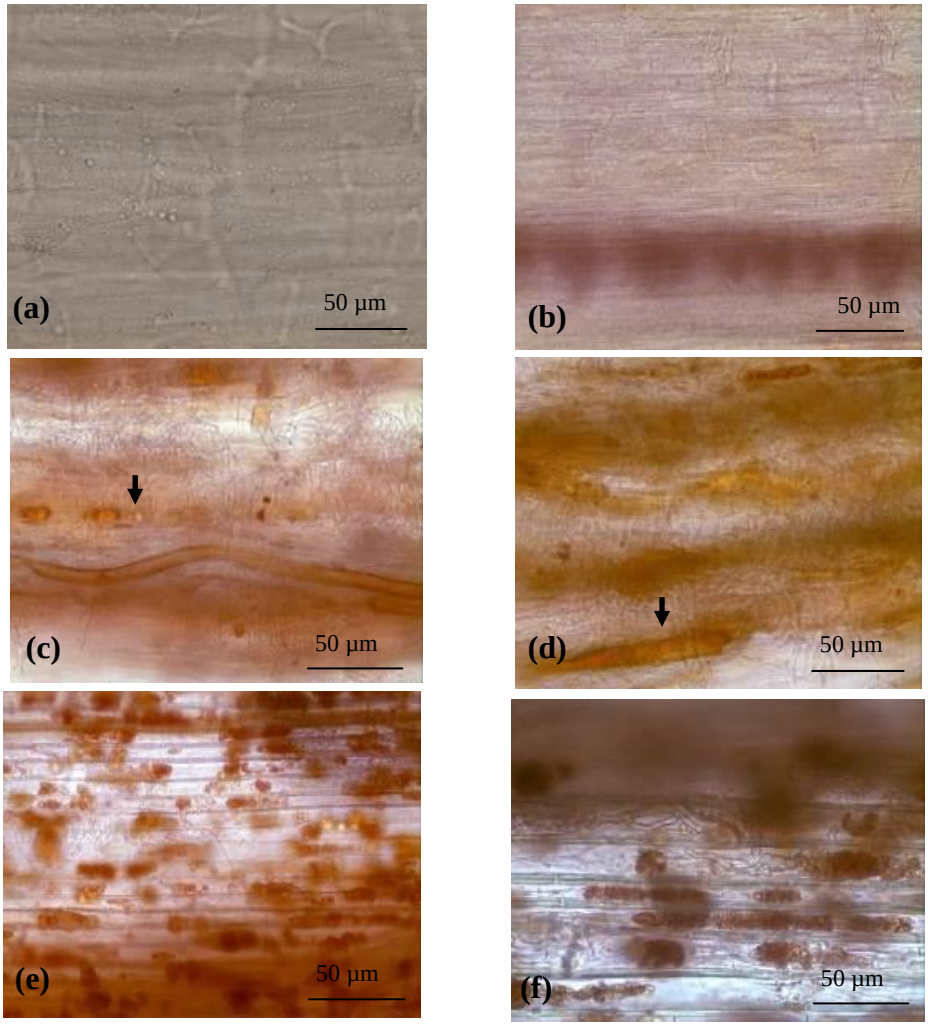

Figure 9. Longitudinal cross section of stem with lignin accumulation marked by purple color (arrow) in *Fusarium subglutinans* infected tissue of tusam *(Pinus merkusii)* seedling.; (a) healthy tusam seedling as a control; (b) two days after inoculation; (c) three days after inoculation; (d) four days after inoculation; (e) five days after inoculation; (f) six, the lignin accumulation invisible due to damage of the seedling tissue (arrow).

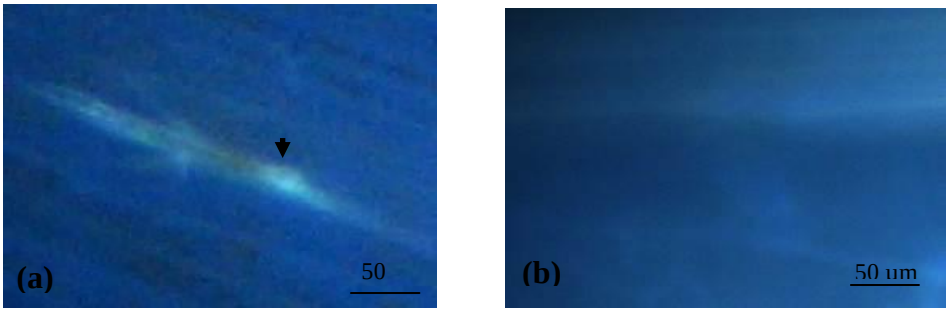

Figure 10. Callose accumulation on *Fusarium subglutinans* infecting tusam (*Pinus merkusii*) seedling tissue; (a) observation using fluorescent microscope Callose accumulation marked with light blue color (arrow); (b) Healhty tusam tissue as a control.

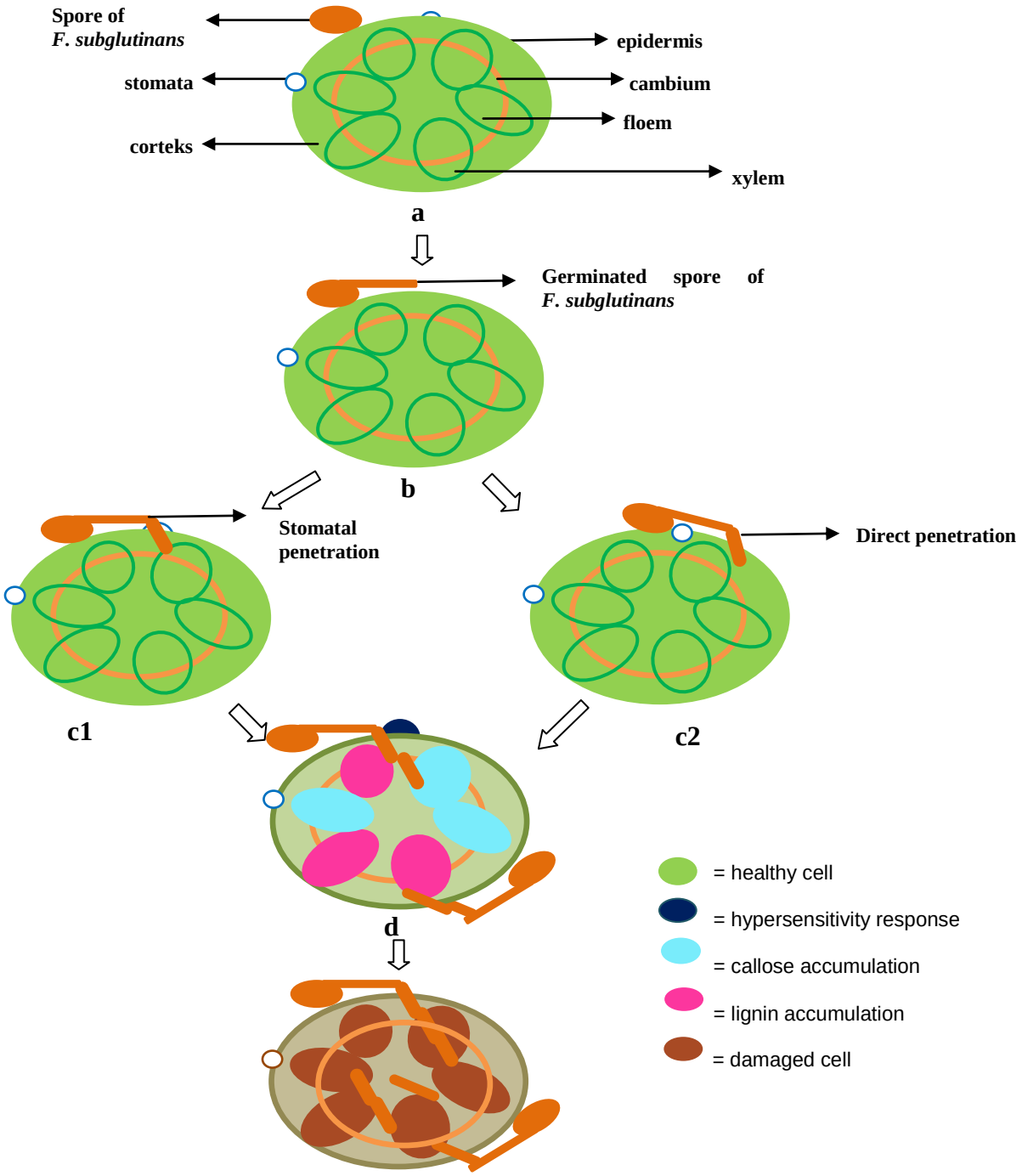

Figure 11. Ilustration of infection process of *F. subglutinans* to tusam (*Pinus merkusii*) seedling on cross section of stem; (a) Spore attached to the stem surface; (b) germinated spore on day three after inoculation; (c1) and (c2) hyphae penetrating the stem tissue on day 4 after inoculation; (d) defence response of tusam seedling occurring on day 5 after inoculation; (e) damage of tusam seedling tissue on day 6 after inoculation

CONCLUSIONS

Penetration process of fungal pathogen into tissue of tusam seedling occured through stomata as well as direct penetration into the plant tissue. Hypersensitivity reaction was present, characterised by lignin and calose accumulation as the response of defence of tusam seedling to the infection of *F. subglutinans*.

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